APPLICATION FOR UNITED STATES PATENT

INVENTOR(S): David G. Heath
Arthur M. Friedlander
George W. Anderson
Susan L. Welkos

INVENTION: RECOMBINANT F1-V PLAGUE VACCINE

SPECIFICATION

SAP 9/94

TITLE OF THE INVENTION

Recombinant F1-V Plague Vaccine

by

David G. Heath
Arthur M. Friedlander
George W. Anderson
Susan L. Welkos

INTRODUCTION

Yersinia pestis is the causative organism of plague in a wide range of animals including man. Bubonic plague in man is most commonly acquired from the bite of an infected flea and is characterized by the swelling of local lymph nodes which form buboes. One of the complications of bubonic plague is secondary pneumonia, and in these cases plague can be transmitted between humans by airborne droplets. Pneumonic plague, particularly, is extremely virulent and results in high mortality rates.

Plague is endemic in regions of North and South America, Africa, China and Asia and, as evidenced by the recent outbreak of pneumonic plague in India [Centers for Disease Control. (1994). Update: Human Plague-India. *MMWR* **43**:722-723], epidemics of enormous consequences remain a potential for this organism. Thus, there is a clear need for a vaccine which would protect individuals living and traveling in endemic areas.

The current human, licensed, vaccines available for prevention of plague are whole cell vaccines. A number of formulations exist. The plague USP vaccine, comprising formaldehyde killed *Y. pestis* bacilli, which is administered to the body via intramuscular injection, produces local and systemic side-effects, ranging from mild headaches to severe malaise and fever. Additionally, the vaccine does not provide complete immunity, since vaccinated individuals can contract pneumonic plague, indicating inadequate immunity at mucosal surfaces.

The live attenuated vaccine EV76 [Meyer *et al.* (1974) *J. Infect. Dis.* **129** suppl., 13-18] was tested extensively and used in the former Soviet Union from 1939, although its

efficacy in evoking an immune response in man is questionable [Meyer *et al.* (1974) *J. Infect. Dis.* **129** suppl., 85-120]. The virulence of EV76 differs in several animal species, and non-human primates are particularly susceptible to a chronic infection with this strain. In the Western World, the vaccine is considered to be unsuitable for mass vaccination due to the severity of the side-effects and the possibility of the strain reverting to full virulence.

Efforts to develop a more effective vaccine have focused on acellular subunits from *Y. pestis* as immunogens. Two of the candidate subunits are the F1 and V antigens. The capsule surrounding *Y. pestis* cells is composed of a protein component known as Fraction 1 (F1) [Baker *et al.*. (1952) *J. Immunol.* **68**: 131-145] which is only fully expressed at 37°C and encoded on the 100 kb pFra plasmid [Protsenko *et al.* (1983) *Genetika* **19:** 1081-1090]. This complex confers resistance to phagocytosis. Detection of antibodies to F1 is the basis of standard serological tests for the surveillance and diagnosis of plague as infected animals and humans produce a strong humoral response to the antigen [Shepherd *et al.* (1986) *J. Clin. Microbiol.* **24**: 1075-1078; Williams *et al.* (1982) *Bull. World Health Organ.* **64**: 745-752].

V antigen, postulated to act as a virulence factor, is a 37 kDa secreted protein which acts as a cytoplasmic regulator of Yops (Yersinia outer membrane protein) expression. The V antigen is encoded on a homologous 75 kb low-calcium response (LCR) plasmid present in *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica.* This plasmid mediates the growth restriction of the organism observed *in vitro* at 37°C in the presence of less than 2.5 mM $Ca2^{+}$. Under such conditions the cells fail to synthesize bulk vegetative proteins although a series of stress proteins and virulence factors are expressed. The V antigen provides both active and passive immunity against experimental infection with $F1^{+}$ strains [Lawton *et al.* (1963) *J. Immunol.* **91:** 179-184; Leary *et al.* (1995) *Infect. Immun.* **63**: 2854-2858; Nakajima *et al.* (1995) *Infect. Immun.* **63**: 3021-3029].

Vaccines which have focused on the capsule protein, F1, expressed either as the purified protein [Baker et al. (1952) *J. Immunol.* **68:** 131-145; Simpson et al. (1990) *Am. J. Trop. Med. Hyg.* **43**: 389-396] or in *aroA* mutant of *Salmonella typhimurium* [Oyston *et al.* (1995) *Infect. Immun.* **63**:563-568] protected mice against virulent *Y. pestis.* However, *Y. pestis* strains lacking or deficient in F1 were isolated from immunized animals after challenge with $F1^+$ organisms from wild rodents [Welkos *et al.* (1995) *Contrib. Microbiol. Immunol.* **13**:299-305] and from a fatal human case of plague [Winter *et al.* (1960) Bull. WHO **23:** 408-409]. Most importantly, $F1^-$ strains are virulent in mice [Worsham *et al.* (1995) *Contrib. Microbiol. Immunol.* **13**: 325-327] and nonhuman primates [Friedlander *et al.* (1995) *Clin. Infect. Dis.* **21:** (Suppl 2), S178-181] and we found in the present study that the human plague vaccine does not protect against infection with $F1^-$ organisms. This implies that, as originally suggested by Burrows [Burrows, T. W. (1957) *Nature* **179:** 1246-1247], an improved plague vaccine protective against both $F1^-$ and $F1^+$ strains of *Y. pestis* must not rely solely on F1.

Therefore, there is a need to develop an improved vaccine protective against both $F1^-$ and $F1^+$ strains of *Y. pestis* suitable for human administration.

SUMMARY

The present invention is directed to a vaccine that satisfies this need. The vaccine of the present invention is protective against both $F1^-$ and $F1^+$ strains of *Y. pestis.* The vaccine of the present invention is composed of a fusion between a portion of the F1 protein and another protective immunogen, the V antigen. This invention is novel because it is a single constructed protein, F1-V, composed of two unique proteins, the entire F1 capsule antigen and V antigen. It induces an immunological response against both F1 protein and V antigen.

The invention was designed to be used in a vaccine affording protection against plague, and to solve the problem of protecting humans against both bubonic and pneumonic plague caused by infection by the subcutaneous (insect bite) and aerosol routes, respectively, with $F1^+$ or $F1^-$ plague organisms, or with strains which may vary in their V antigen.

The advantages of using this protein over the present whole cell vaccine are as follows:

- The current licensed vaccine does not protect mice against subcutaneous challenge with $F1^-$ strains of *Y. pestis*, which have been shown to cause fatal disease in both humans and experimental animals infected by a peripheral, non-respiratory route. The new F1-V vaccine does protect mice against bubonic plague caused by subcutaneous challenge (insect bite) with $F1^-$ organisms.

- The current licensed vaccine does not protect mice against pneumonic plague induced by aerosol challenge with $F1^-$ strains of *Y. pestis*. The new F1-V vaccine does protect mice against pneumonic plague caused by aerosol challenge with $F1^-$ strains.

- The current licensed vaccine does not protect mice against pneumonic plague when challenged by the respiratory route with $F1^+$ strains of *Y. pestis*. The new F1-V vaccine does protect mice against pneumonic plague caused by aerosol challenge with $F1^+$ strains.

- The new F1-V vaccine is expected to protect humans against pneumonic plague produced by strains of *Y. pestis*, either naturally occurring or genetically engineered, which may be altered in their content or composition of V antigen, but which still contain F1. This is because the F1-V vaccine also contains F1. The current licensed vaccine does not protect against pneumonic plague induced by either $F1^-$ or $F1^+$ organisms when given by the aerosol route.

- The new F1-V vaccine is composed of two antigens, both of which have been shown to be protective. The combination of both antigens should provide better protection

against $F1^+$ strains than either F1 or V when used alone as vaccines. This is possible because the immunity induced by F1 and by V occur by different mechanisms which may be additive or synergistic.

- Approximately 8% of humans immunized with the current licensed human plague vaccine fail to develop an immune response to F1 [Marshall *et al.* (1974) *J. Inf. Dis.* **129**:S26-S29]. These non-responders may well be at risk for development of plague. The inclusion of two different protective antigens in the same vaccine will help to eliminate the problem of non-responders to either of the individual antigens and so increase the overall efficacy of vaccination in a human population.

- The new F1-V vaccine is composed of highly purified recombinant proteins which are very well defined. This contrasts with the present human licensed vaccine composed of whole bacteria. The nature of the protective immunogen(s) in the present licensed vaccine is completely unknown. The present licensed vaccine is known to contain and induce antibodies to F1 but it does not induce antibodies to V antigen in mice, suggesting that V antigen is absent.

- The F1-V protein was constructed so that a single protein could be purified as a vaccine component rather than having to produce and purify F1 and V antigen separately. The purification of a single protein as opposed to two separate proteins could result in considerable savings when manufacturing a vaccine.

Therefore, it is an object of the present invention to provide a *Y. pestis* DNA fragment encoding 1563 bp of a fusion protein comprising the F1 protein fused at its carboxyl terminus to the amino terminus of the entire V antigen useful in the production of a diagnostic agent and a vaccine.

It is another object of the present invention to provide an amino acid sequence for *Y. pestis* F1-V protein encoding 521 amino acids.

It is another object of the invention to provide a recombinant vector comprising a vector and the above described DNA fragment for use as a DNA vaccine.

It is a further object of the present invention to provide a host cell transformed with any of the above-described recombinant DNA constructs for use as a live bacterial vaccine when the host cell is a bacteria such as Salmonella, BCG, or a live viral vaccine when the host cell is a virus such as adenovirus, or Venezuelan Equine Encephalitis virus. These transformed cells, bacteria and viruses can also be used as a source for the *Y. pestis* F1-V protein.

It is another object of the present invention to provide a method for producing *Y. pestis* F1-V fusion protein which comprises culturing a host cell under conditions such that a recombinant vector comprising a vector and the *Y. pestis* F1-V protein DNA fragment is expressed and F1-V protein is thereby produced, and isolating F1-V protein for use as a vaccine or a diagnostic agent.

It is still another object of the invention to provide a purified *Y. pestis* F1-V protein useful as a vaccine and a diagnostic agent.

It is a further object of the present invention to provide an antibody to the above-described F1-V protein for use as a therapeutic agent and a diagnostic agent.

It is yet another object of the invention to provide a *Y. pestis* vaccine comprising a F1-V protein effective for eliciting an antigenic and immunogenic response resulting in the protection of a mammal against *Y. pestis* infection by subcutaneous and aerosol route.

It is yet another object of the present invention to provide a method for the diagnosis of *Y. pestis* infection comprising the steps of:

(i) contacting a sample from an individual suspected of having the infection with antibodies which recognize F1-V protein; and

(ii) detecting the presence or absence of a complex formed between *Y. pestis* F1 and/or V antigen and antibodies specific therefor.

It is a further object of the present invention to provide a diagnostic kit comprising a F1-V protein antibody and ancillary reagents suitable for use in detecting the presence *Y. pestis* in mammalian sputum, serum, or tissues.

It is yet another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of infection by *Y. pestis* and other species of *Yersinia* such as *Y. enterocolitica and Y. pseudotuberculosis*, said method comprising providing to an individual in need of such treatment an effective amount of sera from individuals immunized with F1-V protein in a pharmaceutically acceptable excipient.

It is further another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of *Yersinia* infection, said method comprising providing to an individual in need of such treatment an effective amount of antibodies against F1-V protein of *Yersinia pestis* and all or a portion of V antigen of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in a pharmaceutically acceptable excipient.

It is still another object of the present invention to provide antigenic epitopes of F1-V protein, which are useful in peptide vaccine design.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1 shows plasmid pF1V showing an in-frame insertion of the F1 and V open reading frames at the *NdeI* and *BamHI* sites of pET19b separated by an inframe *EcoRI* site. F1-V was transcribed by using the T7 promoter (arrow) within pET19b after induction of T7 polymerase by isopropyl-β-D-thiogalactopyranoside (IPTG). The F1-V amino acid sequence begins with a His-Tag and enterokinase cleavage site derived from pET19b before the methionine start codon of F1. The F1 portion consists of 170 amino acids followed by two amino acids, asparagine and glutamine (encoded by the *EcoRI* site) and the entire sequence of the V antigen. F1-V, therefore, has 521 amino acids with a predicted molecular mass of 57,926 daltons.

Figure 2 shows the gel electrophoresis of F1-V fusion protein. The F1-V fusion protein expressed from pET19b was isolated with 6M urea as recommended (Novagen). Residual endotoxin was removed by passing F1-V over an endotoxin removing gel column (Pierce, Rockford, IL). After the column, F1-V contained 202 endotoxin units per mg of protein by Limulus amoebocyte lysate assay (Sigma, St. Louis, MO) (1 endotoxin unit = 0.1 ng of *E. coli* 055:B5 LPS standard). Recombinant F1 and V were cloned separately and purified as described in Andrews *et al.* (1996) *Inf. Immun.* **64**:2180-2187. F1-V, V and F1 proteins (2μg each) were subjected to SDS-PAGE on 10% tricine gels (Novex, San Diego, CA) and visualized by Coomassie brilliant blue staining.

DESCRIPTION

In one embodiment, the present invention relates to a DNA or cDNA segment which encodes *Y. pestis* recombinant F1-V protein consisting of the F1 protein fused at its carboxyl terminus to the amino terminus of the entire V antigen. The sequence of the 1563 nucleotide DNA segment is specified in SEQ ID NO: 1.

DNA or polynucleotide sequences to which the invention also relates include fragments of F1 or V containing protective epitopes [Motin *et al.* (1994) *Infect Immun.* **62**:4192-4201].

The derived polynucleotide is not necessarily physically derived from the nucleotide sequence shown in SEQ ID NO: 1, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the sequence of bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use. The sequences of the present invention can be used in diagnostic assays such as hybridization assays and polymerase chain reaction assays for the detection of F1 or V sequences of *Y. pestis*.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid such as pET19b, pMBac/pPBac, pSSV1 or any broad host range expression vector such as viral vectors such as adenovirus or Venezuelan Equine Encephalitis virus and others known in the art.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic such as *Bacillus* or *E. coli*, or eukaryotic such as *Saccharomyces or Pichia*, or mammalian cells or insect cells. The vector containing the F1-V protein sequence is expressed in the bacteria and the expressed product used for diagnostic procedures or as a vaccine. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a highly purified IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of F1-V protein. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein described below.

In another embodiment, the present invention relates to a DNA sequence incorporated into a vector which can be used as a DNA vaccine in animals, including humans, or which can be used in a live bacterial or viral vaccine, e.g. *Salmonella*, BCG, adenovirus, or Venezuelan Equine Encephalitis virus.

In another embodiment, the present invention relates to a *Y. pestis* F1-V fusion protein having an amino acid sequence corresponding to SEQ ID NO: 2 and encompassing 521 amino acids or any allelic variation thereof.

A polypeptide or amino acid sequence derived from the amino acid sequence in SEQ ID NO:2, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. The present invention relates to a F1-V protein where the F1 is fused by its carboxy-terminus to the amino-terminus of V antigen constructed

with a polyhistidine and enterokinase site and where the F1 signal sequence is present. Also embodied in this invention is a F1-V protein wherein the F1 is fused to the amino terminus of V, with or without any of the polyhistidine, enterokinase sites and the F1 signal sequence. In addition, a linker of additional amino acids can be fused in frame between the F1 and V antigen (or V antigen and F1) sequences or fragments thereof for convenience as long as the changes do not affect the immunological activity of the fusion protein.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence, or the sequence in SEQ ID NO:1; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides for the purposes of transport or for secretion from the cell or for increasing the protective efficacy in a vaccine. Some examples include the *Yersinia* outer proteins (Yops) of *Yersinia* species or fragments thereof, or the amino-terminal protective antigen binding domain of anthrax toxin lethal and edema factors to name a few.

In a further embodiment, the present invention relates to a method of producing F1-V protein which includes culturing the above-described host cells, under conditions such that the DNA fragment is expressed and F1-V is produced. F1-V can then be isolated using methodology well known in the art or by the production method described below. F1-V protein can be used as a vaccine for immunity against infection with the *Y. pestis* or as a diagnostic tool for detection of *Y. pestis* infection. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit *Y. pestis* , such as host proteins or chemically derived agents or other proteins which may interact with the bacteria to down-regulate or alter the expression of F1 protein or V antigen.

In another embodiment, the present invention relates to antibodies specific for the above-described F1-V protein. For instance, an antibody can be raised against the complete F1-V or against a portion thereof. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to F1-V of the present invention, or a unique portion thereof. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and Practice, Chapter 4, 1986). In addition, the protein or polypeptide can be fused to or combined with other proteins or polypeptides or adjuvants which increase its antigenicity, thereby producing higher titers of neutralizing antibody when used as a vaccine. Examples of such proteins or polypeptides include cholera toxin B subunit and any adjuvants or carriers safe for human use, such as aluminum hydroxide.

In a further embodiment, the present invention relates to a method of detecting the presence of *Y. pestis* infection or antibodies against *Y. pestis* in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), all or a unique portion of the F1-V protein described above, and contacting it with the serum of a person suspected of having plague. The presence of a resulting complex formed between F1-V protein and antibodies specific for either F1 or V in the serum can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of bubonic and pneumonic plague.

Similarly, antibodies to F1-V protein can be used in a rapid diagnostic assay to detect the presence of F1 and/or V antigen in the serum of patients infected with *Yersinia pestis*. Such a test may also be of value in the rapid diagnosis of infection in humans or animals with other *Yersinia* species by detection of V antigen in serum or tissue samples.

The ability of an individual to fight *Y. pestis* infection is dependent on the individual's ability to produce antibodies against *Y. pestis*. Diagnostic assays, similar to those described above, designed to measure the production of protective antibodies against F1-V can be used to measure an individual's response to receiving a plague vaccine.

In another embodiment, the present invention relates to a diagnostic kit which contains F1-V protein from *Y. pestis* and ancillary reagents that are suitable for use in detecting the presence of antibodies to *Y. pestis, Y. enterocolitica and Y. pseudotuberculosis* in serum or a tissue sample. Tissue samples contemplated can be rodents and human, or other mammals. Ancillary reagents would include standard anti-rodent or anti-human antibodies.

In another embodiment, the present invention relates to a vaccine for protection against *Y. pestis* infections by aerosol or subcutaneous route (insect bite). The vaccine comprises F1-V protein, or an immunogenic portion thereof, from a specific strain or species of *Yersinia pestis*. It could also contain V antigen from *Y. enterocolitica* and *Y. pseudotuberculosis*. The vaccine can be prepared by inducing expression of a recombinant expression vector comprising F1-V protein sequence and purifying the resulting protein. The purified F1-V protein is prepared for administration to mammals by methods known in the art, which can include filtering to sterilize the solution, diluting the solution, adding an adjuvant and stabilizing the solution. The vaccine can be lyophilized to produce a vaccine against *Y. pestis* in a dried form for ease in transportation and storage. Further, the vaccine may be prepared in the form of a mixed vaccine which contains the F1-V protein described above and at least one other antigen as long as the added antigen does not interfere with the effectiveness of the vaccine and the side effects and adverse reactions are not increased additively or synergistically.

The vaccine may be stored in a sealed vial, ampule or the like. The present vaccine can generally be administered in the form of a liquid or suspension. In the case where the vaccine is in a dried form, the vaccine is dissolved or suspended in sterilized distilled water before administration. Generally, the vaccine may be administered orally, subcutaneously, intradermally or intramuscularly but preferably intranasally in a dose effective for the production of neutralizing antibody and protection from infection or disease.

In another embodiment, the present invention relates to a method of reducing *Y. pestis* infection symptoms in a patient with bubonic or pneumonic plague by administering to said patient an effective amount of F1-V protein antibodies including those made in humans, either polyclonal or combinations of monoclonals to F1 and V antigen, as described above. When providing a patient with F1-V antibodies, the dosage administered will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds which is in the range of from about 1 pg/kg to 500 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

The following Materials and Methods were used in the Examples below.

DNA methods. All restriction enzymes used were purchased from Life Technologies (Gaithersburg, Md.). Plasmid DNA samples were purified using a Qiagen

plasmid purification kit (Qiagen, Inc., Chatsworth, Calif.) All oligonucleotide primers were synthesized on an Applied Biosystems model 391 DNA synthesizer (Foster City, Calif.) and the polymerase chain reaction (PCR) was performed using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.). DNA sequencing was performed by the dideoxy-chain termination method [Sanger *et al.* (1977) Proc. Natl. Acad. Sci. **74**: 5463-5467] using [α-^{35}S]dATP (Amersham, Arlington Heights, Ill.). Genetic manipulations were performed by standard procedures [Sambrook *et al.* (1989) <u>Molecular Cloning; A Laboratory Manual, 2nd Ed.</u> Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.].

Analytical methods. Protein content was determined by the bicinchoninic acid-Lowry method with BSA as a standard. (Pierce). Purified F1-V was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10% tricine gels (Novex, San Diego, Calif.) and visualized by Coomassie blue R-250 (Bio-Rad Laboratories, Hercules, Calif.) staining.

<u>EXAMPLE 1</u>

Construction, purification, and characterization of recombinant F1-V fusion protein.

To first isolate the F1 structural gene minus its stop codon, plasmid pYPR1 (kindly provided by T. Schwan, Rocky Mountain Laboratories, Hamilton, Mont.) containing the F1 operon [Simpson *et al.* (1990) *Am. J. Trop. Med. Hyg.* **43**:389-396] was used as template DNA in a PCR using as the forward oligonucleotide primer, GGCGCGG<u>CATATG</u>AAAAAAATCAGTTCC (SEQ ID NO:3), containing an internal *Nde* I restriction site (underlined), and the reverse primer, CTC<u>GAATTC</u>TTGGTTAGATACGGT (SEQ ID NO:4), containing an internal *Eco* R1 site (underlined). The V antigen gene was then isolated by PCR using plasmid DNA from ~~a~~ the

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C1 ~~pigmentation negative derivative (Pgm⁻) of *Y. pestis* CO92~~ Antigua strain of Y. pestis, the forward oligonucleotide primer, CGCGAATTCATGATTAGAGCCTACGAA (SEQ ID NO: 5), containing an internal *Eco* RI site (underlined), and the reverse primer, CGCGGATCCTCATTTACCAGACGTGTCA (SEQ ID NO:6) containing an internal *Bam* HI site (underlined). The purified F1 PCR product was then digested with *Nde* I and *Eco* RI while the V antigen purified PCR product was digested with *Eco* RI and *Bam* HI. Both restricted fragments were then ligated to the *Nde* I and *Bam* H1 digested expression vector pET19b (Novagen, Madison, Wis.) and used to transform *Escherichia coli* strain BLR (Novagen) to create plasmid pF1V. The final protein contains an amino-terminal 10 histidines and enterokinase site from pET19b followed by the F1-V protein. The F1 portion consists of 170 amino acids followed by two amino acids, glutamic acid and phenylalanine (the *Eco* RI site) and the entire sequence of the V antigen. F1-V, therefore, has 521 amino acids with a predicted molecular mass of 57,926 daltons. The nucleotide sequence of the F1-V portion of pF1V was verified by sequencing. Two nucleotide differences were found between the V sequence present in F1-V and that reported previously for the V antigen [Price *et al.* (1989) *J. Bacteriol.* **171**: 5646-5653]. A G was replaced by an A at base 247 of the open reading frame, resulting in a change from an alanine to a threonine. At base 324, a G was replaced with a C with no change in the amino acid at that site.

The F1-V fusion protein expressed from pET19b was isolated with 6 M urea as recommended (Novagen). Residual endotoxin was removed by passing F1-V over an endotoxin removing gel column (Pierce, Rockford, Ill.). After the column, F1-V contained 202 endotoxin units per mg of protein by Limulus amoebocyte lysate assay (Sigma, St. Louis, Mo.) (1 endotoxin unit = 0.1 ng of *E. coli* 055:B5 LPS standard).

The recombinant F1-V fusion protein constructed in pET19b consists of the F1 protein fused at its carboxyl terminus to the amino terminus of the entire V antigen. F1-V had a relative molecular weight of 58,000 on SDS-PAGE (Fig. 2) which agreed with its

predicted size of 57,926. The F1-V protein reacted on Western blot with both rabbit polyclonal antibody to F1 as well as mouse monoclonal antibodies directed against V antigen (data not shown). Recombinant F1 and V antigen are also shown in Fig. 2.

After establishing that F1-V had a relative molecular weight consistent with its DNA coding sequence and contained both F1 and V specific epitopes, we tested its ability to protect mice against plague.

EXAMPLE 2

Animal immunization and challenge with *Y. pestis*. Groups of female 8-10 week old Swiss Webster (Hsd:ND4) mice (Harlan Sprague Dawley, Indianapolis, Ind.) were immunized subcutaneously on days 0 and 28 with 0.2 ml of the F1-V, F1, or V antigen preparation adsorbed to the aluminum hydroxide adjuvant, Alhydrogel (1.3%, Superfos Biosector, Vedbaek, Denmark, 0.19 mg aluminum per dose), the human whole-cell plague vaccine U. S. Pharmacopeia (USP) (Greer Laboratories, Lenoir, NC) or Alhydrogel alone as a control. Serum obtained on day 58 after initial immunization was assayed for anti-F1 and anti-V IgG antibody by standard ELISA on individual animals and group geometric mean titers determined. Titers were determined as the reciprocal of the maximum dilution giving an absorbance greater than 0.1 units after subtraction of nonspecific binding in normal serum.

The immunized animals were then challenged on day 78 by either the subcutaneous or aerosol route with wild-type F1$^+$ *Y. pestis*, CO92 (kindly provided by T. Quan, Center for Disease Control, Ft. Collins, Colo.) or C12, an F1$^-$ isogenic derivative of CO92 with a deletion in the F1 structural gene [Worsham *et al.* (1995) *Contrib. Microbiol. Immunol.* **13**: 325-327]. The inocula for s.c. and aerosol challenge were prepared and the animals challenged by s.c. and aerosol routes as previously described [Welkos *et al.* (1995) *Contrib. Microbiol. Immunol.* **13**: 299-305]. The s.c. LD_{50} is 9.1 and 1.9 CFU for F1$^-$

C12 (37) and F1$^+$ C092 (32) strains, respectively. The aerosol LD_{50} is 1.1×10^5 and 2×10^4 CFU (32) for F1$^-$ C12 and F1$^+$ C092 strains, respectively.

All animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. 1985. Guidelines for the care and use of laboratory animals revised. National Institutes of Health publication no. 86-23. National Institutes of Health, Bethesda, MD) and animals were provided food and fresh water ad libitum during the experiment.

Efficacy of F1-V against bubonic plague.

In two separate experiments (Table 1), mice immunized with 13.6 μg of F1-V were protected (90-100% survival) against a subcutaneous challenge with a moderate (57 LD_{50}) or high (1.1×10^6 LD_{50}) dose of the F1$^-$ *Y. pestis* strain, C12, while all control animals died. Another group of animals immunized with 27.2 μg of F1-V all survived (100%) the high-dose challenge. As expected, F1 alone, when adsorbed to Alhydrogel, did not protect animals against challenge with the F1$^-$ C12 strain. Animals given 10 μg of V were afforded the same degree of protection (90% survival) as with F1-V, against the high-dose challenge. Thus, the protective efficacy of the F1-V fusion protein against infection with an F1$^-$ *Y. pestis* strain was equivalent at this challenge dose, to that provided by V alone. In marked contrast, in a separate experiment, the current human, whole-cell plague vaccine USP, failed to protect against a low dose challenge; none of nine challenged animals survived.

TABLE 1 Efficacy of F1-V vaccination against a lethal subcutaneous *Y. pestis* infection of mice.

Treatment Group	Strain	Challenge dose[a]	Survivors/total
Alhydrogel alone	$F1^-$ C12	57	0/10
13.6 μg F1-V	"	57	10/10
10 μg F1	"	60	0/10
Plague USP[b]	"	124	0/9
Alhydrogel alone	"	$1.1x10^6$	0/10
13.6 μg F1-V	"	$1.1x10^6$	9/10
27.2 μg F1-V	"	$1.1x10^6$	10/10
10 μg V	"	$1.1x10^6$	9/10

[a]Number of LD_{50}

[b]The licensed, human, whole-cell plague vaccine.

Efficacy of F1-V against pneumonic plague.

We next determined the efficacy of F1-V against pneumonic plague induced by an aerosol challenge (Table 2). Mice immunized with 13.6 μg of F1-V were completely protected (100% survival) against a moderate (91 LD_{50}) or high (590 LD_{50}) aerosol dose of the $F1^-$ *Y. pestis* strain, C12. Animals given 27.2 μg of F1-V were also completely protected against the high-dose challenge. Similarly, V protected animals exposed to the high-dose aerosol challenge, with 80% of animals surviving. However, as with the s.c. challenge, the plague vaccine USP failed to protect against fatal pneumonic plague; none of eight challenged animals survived. Thus, the whole-cell plague vaccine USP failed to protect mice against challenge with the $F1^-$ strain by either the s.c. or aerosol route, while it does protect against s.c. challenge [Andrews *et al.* (1996) *Infect. Immun.* **64**:2180-2187;

Simpson *et al.* (1990) *Am. J. Trop. Med. Hyg.* **43**:389-396] and partially protects with prolongation of time to death against aerosol challenge with $F1^+$ strains [Andrews *et al.*, *ibid.*; Pitt *et al.* (1994) Abstr. E-45. In: Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.; Smith and Packman (1966) *Brit. J. Exp. Path.* **47**: 25-34]. This differential protection against $F1^+$ and $F1^-$ strains and the absence of an immune response to V antigen in the plague vaccine USP group (Table 2) in agreement with other studies [Chen *et al.* (1961) *J. Immunol.* **87**:64-71; Williamson *et al.* (1995) *FEMS Immunol. Med. Microbiol.* **12**:223-230], strongly suggest that the major protective immunogen in the plague vaccine USP is F1 and that V antigen and other possible immunogens are absent.

The F1-V also protected (100% survival) against a high-dose aerosol challenge with the $F1^+$ *Y. pestis*, CO92 strain isolated from a fatal human pneumonic case. While these studies were in progress, a report described the increased effectiveness of co-immunization with F1 and V antigen in protecting against subcutaneous challenge with an $F1^+$ strain [Williamson *et al.*, *ibid.*]. No studies were performed with $F1^-$ strains or against an aerosol challenge.

TABLE 2. Efficacy of F1-V vaccination against a lethal aerosol *Y. pestis* infection of mice

Treatment Group	Strain	Challenge dose[a]	Survivors/Total	Geometric mean antibody titer[b] F1	V
Alhydrogel alone	$F1^-$ C12	91	0/9	NT[c]	NT
13.6 μg F1-V	"	91	10/10	NT	NT
Alhydrogel alone	"	590	0/14	<640	<640

TABLE 2 Continued. Efficacy of F1-V vaccination against a lethal aerosol *Y. pestis* infection of mice

Treatment Group	Strain	Challenge dose[a]	Survivors/Total	Geometric mean antibody titer[b] F1	V
13.6 μg F1-V	"	590	10/10	66,540	432,376
27.2 μg F1-V	"	590	10/10	108,094	432,376
10 μg V	"	590	8/10	NT	655,360
Plague USP[d]	"	590	0/8	55,738	<640
Alhydrogel alone	$F1^+$ CO92	761	1/10	NT	NT
13.6 μg F1-V	"	761	10/10	NT	NT

[a]Number of LD_{50}

[b]Serum obtained on day 58 after the initial vaccine dose was assayed for anti-F1 and anti-V IgG antibody by ELISA on individual animals and group geometric mean titers determined as described in Materials and Methods.

[c]Not tested.

[d]The licensed, human, whole-cell plague vaccine.

Our results clearly demonstrate that mice immunized with a fusion protein, consisting of the F1 capsular antigen fused at its carboxyl terminus to the amino terminus of the V antigen of *Y. pestis*, were provided with excellent protection against both parenteral and aerosol challenge with an $F1^-$ *Y. pestis* strain. The F1-V fusion protein also protected mice against an aerosol challenge with an $F1^+$ *Y. pestis* strain. Other workers, as noted previously, showed that V antigen protects animals against challenge with virulent

F1⁺ strains, results which we have confirmed and extended to aerosol challenge. This raises the issue of whether the F1 portion of F1-V is immunogenic and contributes to the protection against challenge with the F1⁺ *Y. pestis* strain CO92. Several lines of evidence suggest that the F1 portion is immunogenic and protective. The development of high levels of antibody to F1 after immunization with F1-V (Table 2) and the numerous reported studies showing a strong correlation between the level of antibody to F1 and protection against infection [Williams and Cavanaugh (1979) Bull. WHO **57**:309-313) suggest the F1 portion of F1-V helps protect against challenge with F1⁺ *Y. pestis*. Further support for a protective role for the F1 portion of F1-V was provided by our observations with a smaller F1-V fusion protein we constructed that consisted of the entire F1 gene fused at its carboxyl terminus to amino acids 168 to 275 of V antigen. This fusion protein was unable to effectively immunize mice against a subcutaneous challenge with the F1⁻ *Y. pestis* C12 strain (3 survivors out of 10 mice challenged with 55 LD_{50}, data not shown), showing that the V segment of the fusion protein was not protective. However, this same fusion protein was able to protect mice against the F1⁺ *Y. pestis* CO92 strain (10 survivors out of 10 mice challenged subcutaneously with 63 LD_{50}, data not shown). These results indicate the F1 portion of this fusion protein was immunogenic, enabling mice to survive challenge against infection with the F1⁺ *Y. pestis* CO92, while the V segment failed to elicit a significant protective immune response against challenge with the V antigen expressing but F1⁻ *Y. pestis* C12 strain. The value of a combined immune response to both F1 and V for combating infection with F1⁺ *Y. pestis* strains is supported by the studies of Burrows and Bacon [Burrows, T. W. (1963) *Ergeb. Mikrobiol. Immunitatsforch. Exp. Ther.* **37**:59-113; Burrows and Bacon (1958) *Brit. J. Exp. Pathol.* **39**: 278-291] who found that serum from rabbits immunized with attenuated strains of *Y. pestis* expressing both F1 and V antigen provided better passive protection in mice against wild type F1⁺ *Y. pestis* than serum from rabbits immunized with attenuated *Y. pestis* strains expressing only V antigen. It is also supported by the recent study showing increased protection by co-immunization

of F1 with V antigen [Williamson *et al., ibid.*]. Better protection by antibody directed against both F1 and V might occur by counteracting both the anti-phagocytic activity associated with F1 [Burrows, T. W., *ibid.*] and the virulence-enhancing activity associated with secreted V antigen [Nakajima *et al.* (1995) *Infect. Immun.* **63**: 3021-3029].

An additional advantage of a vaccine containing both F1 and V is that F1 should protect against variant strains of *Y. pestis* which might be altered in the amount or composition of V antigen, in a manner analogous to that by which V protects against $F1^-$ strains. Indeed, isolates deficient in V, determined immunologically, have been cultured from immunized animals infected with V-containing, wild-type *Y. pestis* [Williams *et al.* (1974) *Trans. Roy. Soc. Trop. Med. Hyg.* **68**:171]. Furthermore, variability in the structural gene for V has been described for *Y. pseudotuberculosis* [Motin *et al.*(1994) *Infect. Immun.* **62**:4192-4201], although to date, this has not been reported for *Y. pestis.*

Another potential advantage of an F1-V multicomponent vaccine would be to protect individuals who may be non-responders to one component of a vaccine. Indeed, some recipients of the current plague vaccine USP fail to develop an antibody response to F1 [Marshall *et al.* (1974) *J. Infect. Dis.* **129**:S26-S29]. A similar approach to the development of recombinant multicomponent vaccines may be of value against other infectious diseases.

In summary, we constructed an F1-V fusion protein to provide optimal protective immunity against pneumonic as well as bubonic plague due to either wild-type $F1^+$ *Y. pestis* or fully virulent $F1^-$ *Y. pestis* strains which may occur naturally or may develop after infection of vaccinated individuals. The new vaccine was shown to be effective against both $F1^-$ as well as $F1^+$ *Y. pestis* strains. Most importantly, it prevented both fatal pneumonic as well as bubonic infection. This vaccine candidate may lead to the development of an improved human plague vaccine.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: David G. Heath
Arthur M. Friedlander
George W. Anderson
Susan L. Welkos

(ii) TITLE OF INVENTION: Recombinant F1-V Plague Vaccine

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: John Moran
(B) STREET: USA MRMC - MRMC-JA
(C) CITY: FORT DETRICK, FREDERICK
(D) STATE: MARYLAND
(E) COUNTRY: USA
(F) ZIP: 21702-5012

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 7.5
(D) SOFTWARE: Microsoft Word 6.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Moran, John
(B) REGISTRATION NUMBER: 26,313
(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION
(A) TELEPHONE: (301) 619-2065
(B) TELEFAX: (301) 619-7714

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1566 bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CAGTTCCGTT	ATCGCCATTG	CATTATTTGG	AACTATTGCA	120
ACTGCTAATG	CGGCAGATTT	AACTGCAAGC	ACCACTGCAA	160
CGGCAACTCT	TGTTGAACCA	GCCCGCATCA	CTCTTACATA	200
TAAGGAAGGC	GCTCCAATTA	CAATTATGGA	CAATGGAAAC	240
ATCGATACAG	AATTACTTGT	TGGTACGCTT	ACTCTTGGCG	280
GCTATAAAAC	AGGAACCACT	AGCACATCTG	TTAACTTTAC	320
AGATGCCGCG	GGTGATCCCA	TGTACTTAAC	ATTTACTTCT	360
CAGGATGGAA	ATAACCACCA	ATTCACTACA	AAAGTGATTG	400
GCAAGGATTC	TAGAGATTTT	GATATCTCTC	CTAAGGTAAA	440
CGGTGAGAAC	CTTGTGGGGG	ATGACGTCGT	CTTGGCTACG	480
GGCAGCCAGG	ATTTCTTTGT	TCGCTCAATT	GGTTCCAAAG	520
GCGGTAAACT	TGCAGCAGGT	AAATACACTG	ATGCTGTAAC	560
CGTAACCGTA	TCTAACCAAG	AATTCATGAT	TAGAGCCTAC	600
GAACAAAACC	CACAACATTT	TATTGAGGAT	CTAGAAAAAG	640
TTAGGGTGGA	ACAACTTACT	GGTCATGGTT	CTTCAGTTTT	680
AGAAGAATTG	GTTCAGTTAG	TCAAAGATAA	AAATATAGAT	720
ATTTCCATTA	AATATGATCC	CAGAAAAGAT	TCGGAGGTTT	760
TTGCCAATAG	AGTAATTACT	GATGATATCG	AATTGCTCAA	800
GAAAATCCTA	GCTTATTTTC	TACCCGAGGA	TACCATTCTT	840
AAAGGCGGTC	ATTATGACAA	CCAACTGCAA	AATGGCATCA	880
AGCGAGTAAA	AGAGTTCCTT	GAATCATCGC	CGAATACACA	920
ATGGGAATTG	CGGGCGTTCA	TGGCAGTAAT	GCATTTCTCT	960

TTAACCGCCG ATCGTATCGA TGATGATATT TTGAAAGTGA 1000

TTGTTGATTC AATGAATCAT CATGGTGATG CCCGTAGCAA 1040

GTTGCGTGAA GAATTAGCTG AGCTTACCGC CGAATTAAAG 1080

ATTTATTCAG TTATTCAAGC CGAAATTAAT AAGCATCTGT 1120

CTAGTAGTGG CACCATAAAT ATCCATGATA AATCCATTAA 1160

TCTCATGGAT AAAAATTTAT ATGGTTATAC AGATGAAGAG 1200

ATTTTTAAAG CCAGCGCAGA GTACAAAATT CTCGAGAAAA 1240

TGCCTCAAAC CACCATTCAG GTGGATGGGA GCGAGAAAAA 1280

AATAGTCTCG ATAAAGGACT TTCTTGGAAG TGAGAATAAA 1320

AGAACCGGGG CGTTGGGTAA TCTGAAAAAC TCATACTCTT 1360

ATAATAAAGA TAATAATGAA TTATCTCACT TTGCCACCAC 1400

CTGCTCGGAT AAGTCCAGGC CGCTCAACGA CTTGGTTAGC 1440

CAAAAAACAA CTCAGCTGTC TGATATTACA TCACGTTTTA 1480

ATTCAGCTAT TGAAGCACTG AACCGTTTCA TTCAGAAATA 1520

TGATTCAGTG ATGCAACGTC TGCTAGATGA CACGTCTGGT 1560

AAATGA 1566

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 521
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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His Ile Asp Asp Asp Asp Lys His Met Lys Lys Ile Ser Ser Val
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Ile Ala Ile Ala Leu Phe Gly Thr Ile Ala Thr Ala Asn Ala Ala
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35 40 45

Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala Thr Leu Val Glu Pro
50 55 60

Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala Pro Ile Thr Ile
65 70 75

Met Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val Gly Thr Leu
80 85 90

Thr Leu Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser Val Asn
95 100 105

Phe Thr Asp Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr Ser
110 115 120

Gln Asp Gly Asn Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys
125 130 135

Asp Ser Arg Asp Phe Asp Ile Ser Pro Lys Val Asn Gly Glu Asn
140 145 150

Leu Val Gly Asp Asp Val Val Leu Ala Thr Gly Ser Gln Asp Phe
155 160 165

Phe Val Arg Ser Ile Gly Ser Lys Gly Gly Lys Leu Ala Ala Gly
170 175 180

Lys Tyr Thr Asp Ala Val Thr Val Thr Val Ser Asn Gln Glu Phe
185 190 195

Met Ile Arg Ala Tyr Glu Gln Asn Pro Gln His Phe Ile Glu Asp
200 205 210

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215 220 225

Val Leu Glu Glu Leu Val Gln Leu Val Lys Asp Lys Asn Ile Asp
230 235 240

Ile Ser Ile Lys Tyr Asp Pro Arg Lys Asp Ser Glu Val Phe Ala
245 250 255

Asn Arg Val Ile Thr Asp Asp Ile Glu Leu Leu Lys Lys Ile Leu
260 265 270

Ala Tyr Phe Leu Pro Glu Asp Thr Ile Leu Lys Gly Gly His Tyr
275 280 285

Asp Asn Gln Leu Gln Asn Gly Ile Lys Arg Val Lys Glu Phe Leu
290 295 300

Glu Ser Ser Pro Asn Thr Gln Trp Glu Leu Arg Ala Phe Met Ala

305 310 315

Val Met His Phe Ser Leu Thr Ala Asp Arg Ile Asp Asp Asp Ile
320 325 330

Leu Lys Val Ile Val Asp Ser Met Asn His His Gly Asp Ala Arg
335 340 345

Ser Lys Leu Arg Glu Glu Leu Ala Glu Leu Thr Ala Glu Leu Lys
350 355 360

Ile Tyr Ser Val Ile Gln Ala Glu Ile Asn Lys His Leu Ser Ser
365 370 375

Ser Gly Thr Ile Asn Ile His Asp Lys Ser Ile Asn Leu Met Asp
380 385 390

Lys Asn Leu Tyr Gly Tyr Thr Asp Glu Glu Ile Phe Lys Ala Ser
395 400 405

Ala Glu Tyr Lys Ile Leu Glu Lys Met Pro Gln Thr Thr Ile Gln
410 415 420

Val Asp Gly Ser Glu Lys Lys Ile Val Ser Ile Lys Asp Phe Leu
425 430 435

Gly Ser Glu Asn Lys Arg Thr Gly Ala Leu Gly Asn Leu Lys Asn
440 445 450

Ser Tyr Ser Tyr Asn Lys Asp Asn Asn Glu Leu Ser His Phe Ala
455 460 465

Thr Thr Cys Ser Asp Lys Ser Arg Pro Leu Asn Asp Leu Val Ser
470 475 480

Gln Lys Thr Thr Gln Leu Ser Asp Ile Thr Ser Arg Phe Asn Ser
485 490 495

Ala Ile Glu Ala Leu Asn Arg Phe Ile Gln Lys Tyr Asp Ser Val
500 505 510

Met Gln Arg Leu Leu Asp Asp Thr Ser Gly Lys
515 520

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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CTCGAATTCT   TGGTTAGATA   CGGT            24
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(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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CGCGAATTCA   TGATTAGAGC   CTACGAA         27
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(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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CGCGGATCCT   CATTTACCAG   ACGTGTCA        28
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